AMPHIBIAN AND REPTILE DISEASES

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Low Historical Prevalence of the Fungal Pathogen *Batrachochytrium dendrobatidis* in Black-spotted Newts (*Notophthalmus meridionalis*) from Texas, USA, and Northeastern Mexico

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Many amphibian species have declined throughout their ranges, and often the causes of those declines are poorly understood (Luedtke et al. 2023). Large-scale amphibian declines were first observed in the 1970s, and in the following decades, *Batrachochytrium dendrobatidis* (Bd) infection was determined as the cause of some of these declines (Fisher and Garner 2020). Retrospective studies allow us to evaluate historical disease patterns and are particularly valuable in species that are extirpated or extinct. The Black-spotted Newt (*Notophthalmus meridionalis*; Salamandridae) is an understudied species of salamander native to south Texas, USA, and northeastern Mexico that has shown alarming population declines over the past 70 years, most notably prior to 1980 (Judd 1985; Rappole and Klicka 1991). *Notophthalmus meridionalis* has disappeared from the northern part of its range in recent decades. Since 2000, individuals have only been found in three (Cameron, Hidalgo, and Willacy) of thirteen Texas counties that historically were part of their range (Robinson et al. 2022). By 2019, the species had been extirpated from central Tamaulipas, southern Veracruz, and northern Puebla, Mexico (IUCN 2022). This species was listed on the IUCN Red List in 2004 as Endangered, and in 2019 was re-evaluated as Vulnerable (IUCN 2022). In Mexico and Texas, it is listed as in Danger of Extinction and Threatened, respectively (SEMARNAT 2010; TPWD 2020; IUCN 2022). *Notophthalmus meridionalis* is a part of the U.S. Fish and Wildlife Service National Listing Workplan, but it is not currently protected under the Endangered Species Act (USFWS 2023). Multiple factors have likely contributed to its decline, including habitat loss, habitat fragmentation, and pesticide use (Judd 1985; Jahrsdoerfer and Leslie 1988; Dixon 2013; Villamizar-Gomez et al. 2021; IUCN 2022; Robinson et al. 2022). In the past century, the majority of native habitat in south Texas has been cleared for agriculture, urbanization, and roads, leaving small patches of fragmented habitat remaining across the species' historical northern range (Jahrsdoerfer and Leslie 1988). However, declines in *N. meridionalis* have also been observed in protected areas not affected by habitat loss and degradation (e.g., Welder Wildlife Refuge, San Patricio County, Texas; Davis et al. 2023). The reason for their disappearance from these areas is unknown, raising the question of whether Bd infections were contributing to their decline. The south Texas and northeastern Mexico ecoregion is not a Bd hotspot and Bd is not predicted to occur at high prevalence according to distribution models (Bolom-Huet et al. 2019; Villamizar-Gomez et al. 2021). Despite this, Bd has been detected in this region in multiple amphibian species (Basanta et al. 2020; Villamizar-Gomez et al. 2021). Importantly, *N. meridionalis* has not been extensively surveyed for Bd infection and its susceptibility to this pathogen has not been evaluated. However, its congeners, *N. viridescens* and *N. perstriatus*, are known Bd reservoirs (Hartmann et al. 2024).

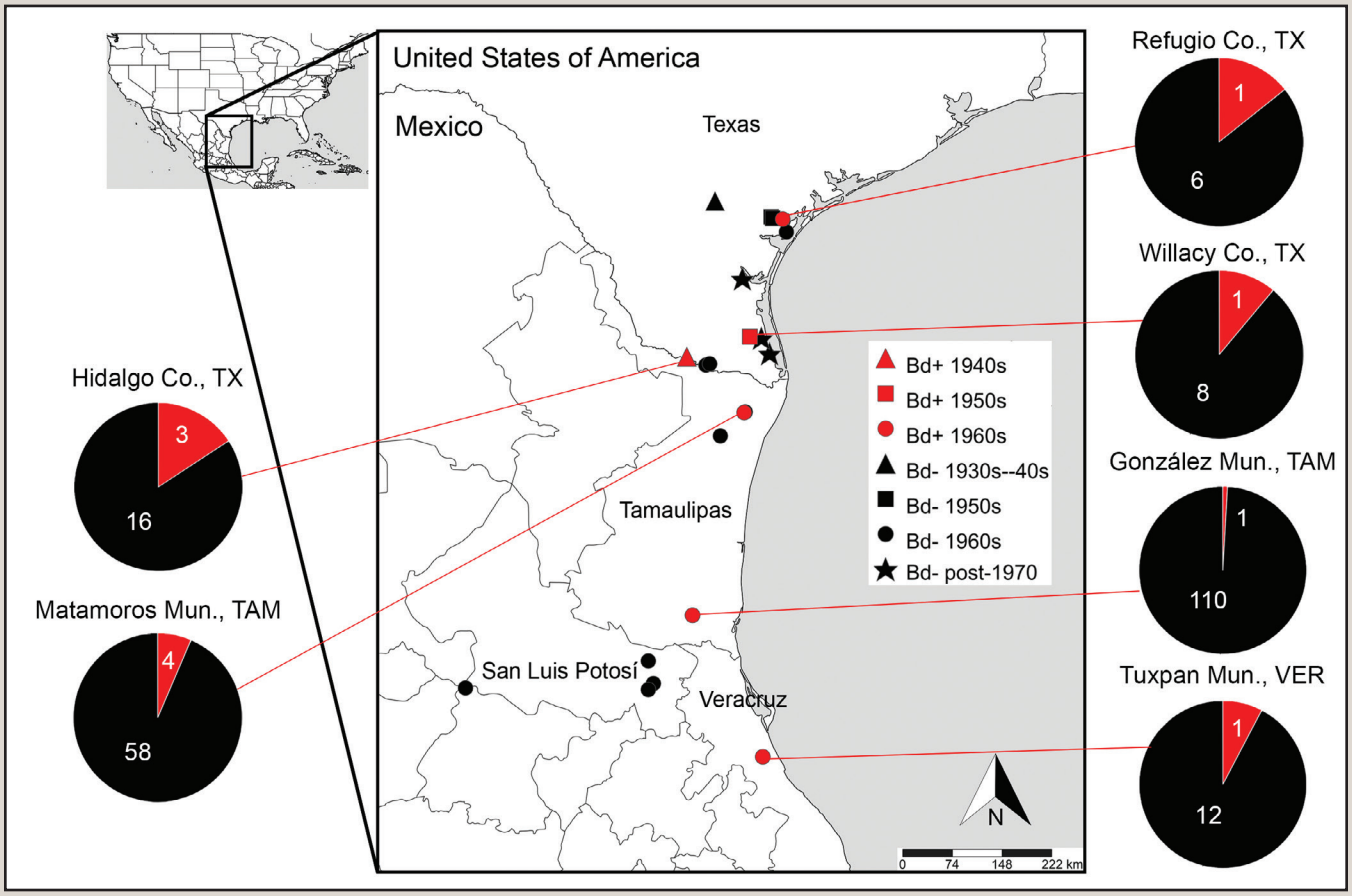

FIG. 1. *Notophthalmus meridionalis* specimen localities, with symbols representing decade collected. Localities with *Batrachochytrium dendrobatidis* positive (Bd+) results from laboratory analyses are broken down into a visual of the number of positive samples (red) versus the number of Bd-negative samples (Bd-; black). Points represent multiple samples, and Bd-positive results were plotted on top of negative results if the localities overlap, see Table 1 for a detailed breakdown of results.

Here, we assayed 314 *N. meridionalis* museum specimens to determine whether the historical presence of Bd may have contributed to population declines in this species (Appendix).

We collected swab samples from all available formalin-fixed museum specimens of *N. meridionalis* from the Biodiversity Collections, The University of Texas at Austin (TNHC), Biodiversity Research and Teaching Collections, Texas A&M University (TCWC), and Collection of Vertebrates, Oklahoma State University (OSU); while specimens of *N. meridionalis* are present in other collections, these three collections were selected because they permitted loans during 2020 when sampling was conducted. A total of 314 specimens were sampled from 23 localities across the range of *N. meridionalis* in the states of Texas (USA), San Luis Potosí (MX), Tamaulipas (MX), and Veracruz (MX), covering the years 1938–2014 (Fig. 1; Table 1). The majority of samples (N = 295) were collected between 1940 and 1968. Specimens were removed from jars, rinsed with clean ethanol or isopropanol (depending on the fluid the specimens were stored in) to remove potential external contaminants prior to sampling. Gloves were changed between animals to further reduce the risk of cross-contamination. Specimens were swabbed using sterile rayon-tipped swabs (MW113, Medical Wire & Equipment, Corsham, UK); following established retrospective Bd study approaches (Cheng et al. 2011; Lannoo et al. 2011; Watters et al. 2016). Swabs were then placed in sterile 1.5-ml microcentrifuge tubes with three drops of ethanol or isopropanol and stored at -80°C until extracted.

We isolated DNA from swabs using a phenol-chloroform-isoamyl protocol, followed by a TaqMan real-time quantitative PCR assay to detect the presence of Bd (Boyle et al. 2004). We used a serial dilution series of synthetic gBlock standards of the Bd ITS region from 10^6–10^1 ITS copies. Samples were run in duplicate, and those that exhibited a typical exponential amplification curve prior to 50 cycles were considered positive. Those that tested positive for Bd in only one replicate were re-run in duplicate and considered positive if at least two of the four replicates amplified.

Bd was detected in 11 of 314 samples, yielding an overall detection prevalence of 3.5% (Table 1). Nine samples amplified at or above 1 ITS copy (range: 1–26 ITS copies), and two samples amplified below 1 ITS copy on average. Most of the Bd-positive specimens (N = 10) were collected in Texas and Tamaulipas between 1940 and 1968 (Table 1; Fig. 1). One specimen tested Bd-positive from Veracruz in 1965.

Based on the low prevalence of Bd in these specimens, it is unlikely to have been a driving factor in the rapid decline of *N. meridionalis* in the mid-20th century. However, our results indicate that Bd was present across this species' range throughout the period of most intensive decline and as early as 1940. It is possible that Bd presence may have exacerbated stressors that contributed to their decline. Further research to identify alternate threats at these localities are necessary to inform *N. meridionalis* conservation. We note that while our

TABLE 1. Examined *Notophthalmus meridionalis* museum specimens by county (Co.; USA) or municipality (Mun.; MX) and by decade. Numbers in cells represent the number of Bd-positive skin swabs/total number of swabs tested for that locality and from that decade.

	Decade						
Location	1930s	1940s	1950s	1960s	1970s	1980s	2010s
Texas, USA							
Cameron Co.	–	–	–	–	0/1	–	–
Hidalgo Co.	0/2	3/19	–	0/2	–	–	–
Kleberg Co.	–	–	–	–	–	0/5	–
Live Oak Co.	0/8	–	–	–	–	–	–
Refugio Co.	–	–	–	1/7	–	–	–
San Patricio Co.	–	–	0/30	0/2	–	–	–
Willacy Co.	–	–	1/9	–	–	–	0/3
San Luis Potosí, Mexico							
Ciudad Valles Mun.	–	–	–	0/31	–	–	–
Villa de Arriaga Mun.	–	–	–	0/2	–	–	–
Tamaulipas, Mexico							
González Mun.	–	–	–	1/111	–	–	–
Matamoros Mun.	–	–	–	4/62	–	–	–
San Fernando Mun.	–	–	–	0/7	–	–	–
Veracruz, Mexico							
Tuxpan Mun.	–	–	–	1/13	–	–	–
Total Prevalence (% by decade)	0/10 (0%)	3/19 (15.8%)	1/39 (2.6%)	7/237 (3.0%)	0/1 (0%)	0/5 (0%)	0/3 (0%)

qPCR assay is sensitive enough to detect even very low-level concentrations of Bd, the true rate of infection among our specimens may be higher because of the potential for DNA to degrade when specimens are fixed in formalin and stored in ethanol long-term (Muletz et al. 2014). However, false negatives likely had low Bd loads upon initial collection. Another caveat of retrospective Bd studies is that cross-contamination may occur (e.g., among individuals within the same collection jar, or during animal collection). While we cannot rule out that this occurred for three of our positive samples that may have been stored in the same jar at TNHC, these specimens represent a single collection event; whether one or three newts were Bd-positive from this site at this time does not change the conclusion that Bd was detected, but incidence was low. An additional threat to amphibian biodiversity is chytridiomycosis caused by *Batrachochytrium salamandrivorans* (Bsal), a second pathogenic *Batrachochytrium* that is spreading throughout Europe and causing notable declines in salamander populations. Although Bsal has not yet been detected in North America, its introduction could be disastrous for salamander biodiversity (Waddle et al. 2020; Olson et al. 2024). Among all salamanders, the family Salamandridae is particularly susceptible to Bsal (Martel et al. 2014; Gray et al. 2023). Future studies are warranted to assess the susceptibility of *N. meridionalis* to Bsal chytridiomycosis, more broadly monitor for Bsal – especially within the range of *N. meridionalis*, develop downscaled models of the probability of Bd and/or Bsal disease outbreaks that may affect populations of this and other salamander species, and broaden utilization of museum specimens (Nachman et al. 2023). These contributions would improve understanding of the global spread of Bd and Bsal and how each may have impacted historical amphibian declines. Retrospective studies of museum specimens provide critical insights into the geographical and historical impacts of diseases. This is one example of how scientific collections provide valuable windows into the past with many applications to conservation. These valuable resources should continue to be maintained long into the future.

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Appendix

List of *Notophthalmus meridionalis* specimens examined by each collection locality. * = indicates a sampling locality with at least one specimen that tested positive for *Batrachochytrium dendrobatidis*. TNHC = Biodiversity Collections, The University of Texas at Austin; TCWC = Biodiversity Research and Teaching Collections, Texas A&M University; OSU = Collection of Vertebrates, Oklahoma State University.

MEXICO: SAN LUIS POTOSÍ: Ciudad Valles Mun.: Cueva Chica, 11.5 mi S Valles (3–4 mi N El Pujal) (21.90700°N, 98.93999°W): TNHC 69227; Cd. Valles, 11.5 mi S (Cueva Chica) (21.82527°N, 99.00755°W): TNHC 84154–84156; Valles, 14.8 mi N (22.20751°N, 99.00755°W): TNHC 84157–84182; Villa de Arriaga Mun.: Arriaga, 6 mi SW Hwy 80 (21.84696°N, 101.44827°W): TNHC 93382, 93383.

TAMAULIPAS: González Mun.: Gonzales, just E on Monte-Tampico Rd; 1 mile SE on Hwy 80 (22.81655°N, 98.41797°W)*: TNHC 84209, 84218–84332; Matamoros Mun.: Matamoros, 28 mi S on 101 (25.52462°N, 97.71472°W): TNHC 84197–84217; Matamoros, 29 mi S [on] Hwy 101 (25.51694°N, 97.72821°W)*: TNHC 84352–84389; San Fernando Mun.: El Tejon, 9.5 mi N (25.20618°N, 98.04629°W): TNHC 84390–84393, 84448–84450.

VERACRUZ: Tuxpan Mun.: Tuxpan, 5 mi WSW (20.92991°N, 97.47989°W)*: TNHC 84183–84195.

USA: TEXAS: Cameron Co.: Laguna Atascosa National Wildlife Refuge (26.30152°N, 97.38986°W): TNHC 52856; Hidalgo Co.: 4 mi S McAllen (26.14935°N, 98.23927°W): TCWC 17546; 5 mi SE McAllen (26.16615°N, 98.18885°W): TCWC 17547; Mission, 10 mi W on Banks of Lake La Jolla (26.25891°N, 98.49434°W)*: TNHC 6120, 6122–6141; Kleberg Co.: 3–5 mi E Riviera (27.29802°N, 97.75221°W): TCWC 64831–64835; Live Oak Co.: M. George W. (28.3325°N, 98.117505°W): OSU A846, A848–A854; Refugio Co.: Bayside (28.09390°N, 97.21471°W)*: TNHC 84147–84149, 84151–84153; San Patricio Co.: Big Lake, 50 ft S on Welder Wildlife Foundation (28.10957°N, 97.36599°W): TNHC 26598, 27813–27837, 27839; Sinton, Welder Wildlife Refuge (28.1057°N, 97.34855°W): TNHC 27811, 27812; 1.3 mi NW 300 degrees W, Aransas Pass (27.92282°N, 97.16510°W: TCWC 97539, 97540; Willacy Co.: San Perlita, 2 mi NNW (26.52802°N, 97.65213°W)*: TNHC 15068, 15069, 15138–15140, 19194, 19195, 101943, 101944; El Sauz Ranch, "Newt Pond" (26.50727°N, 97.49904°W): TCWC 98147.